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Research Article

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Decreased Content and Surface Expression of α-Granule Membrane Protein GMP-140 in One of Two Types of Platelet αδ Storage Pool Deficiency

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Abstract

To determine whether α-granule membranes are present in platelets of patients with storage pool deficiencies of both alpha and dense granules (αδ-SPD), we examined the content and surface expression of the α-granule membrane protein GMP-140 in one patient (J.C.) with a severe α-granule deficiency and in three members of a family (family C) with milder α-granule deficiencies. Surface expression of GMP-140 in stimulated platelets, assessed by flow cytometric measurements of the binding of two anti-GMP-140 monoclonal antibodies, was 24–38% of normal values in platelets from patient J.C., vs. 60–95% of normal values in family C. Total platelet content of GMP-140, determined in platelet lysates by antigen-capture ELISA, was 49% of normal in patient J.C., but normal in the members of family C. Platelets of patient J.C. were found to be heterogeneous with respect to GMP-140 content and surface expression by both flow cytometry and immunogold electron microscopy. Approximately 80% of her platelets expressed little or no GMP-140 after stimulation, whereas the remaining 20% expressed normal amounts of GMP-140 and showed extensive immunogold labeling of typical α-granules and clear vacuoles. No such heterogeneity was found in platelets from family C. These findings in the severe αδ-SPD patient are in clear contrast to the observations of normal GMP-140 content in the three other αδ-SPD patients, and in patients with the gray platelet syndrome, reported previously by others. These results illustrate the phenotypic heterogeneity of α-granule deficiencies in human platelets, and suggest that a defect in granule formation in the megakaryocytes may account for the α-granule defect in at least one form of αδ-SPD. (*J. Clin. Invest.* 1991. 87:919–929.) Key words: α-granule membrane • platelet storage pool deficiency

Introduction

Platelet storage pool deficiencies (SPD)[1] comprise a range of disorders encompassing variable degrees of reduction in the numbers and contents of dense granules, α-granules, or both types of granules (1). In addition to the more frequently occurring disorders of dense granules only (δ-SPD), two such disorders involving α-granule deficiencies have been characterized: αδ-SPD, which includes patients with marked deficiencies of dense granules as well as more variable deficiencies of α-granules (1), and α-SPD, or the gray platelet syndrome (2), in which severe reductions occur only in the α-granules and their contents (3–5). Both disorders are associated with impaired platelet function as indicated by decreased aggregation responses. In α-SPD, aggregation to ADP, collagen, and thrombin is decreased (4, 5), whereas in αδ-SPD both primary and secondary aggregation responses to most of the common agonists are severely impaired (6–8). In addition, thromboxane formation via cyclooxygenase (7), and platelet adhesion and thrombus formation on subendothelium (9) are also markedly decreased in αδ-SPD.

The nature of the granule defects in almost all types of congenital SPD remains to be determined. Typical dense granules and/or α-granules are reduced in number or absent in electron micrographs of SPD platelets (1, 10, 11), but this does not distinguish between the possibilities of a reduction in, or absence of, the granule structure itself, or the presence of an abnormal granule structure which may be incapable of incorporating or retaining some or all of its constituents. A third alternative, the presence of a normal granule structure, but an impairment in the delivery of constituents to the developing granules in the megakaryocyte, is also a possibility.

One means of examining these hypotheses in SPD involving the α-granules may be provided by the identification of an α granule membrane protein called GMP-140, or PADGEM, in platelets and megakaryocytes (12–14) as well as in endothelial cells (15, 16) and human erythroleukemia cells (17, 18). In platelets, this integral membrane protein is specific to the membranes of α-granules. As shown by studies using both monoclonal and polyclonal antibodies to GMP-140, this protein is redistributed to the platelet surface and the surface-connected canalicular system following stimulation (12, 13), and has been found to mediate the adhesion of stimulated platelets to neutrophils and monocytes (19, 20). Thus, measurement of GMP-140 content and surface expression could provide an indication of whether α-SPD and αδ-SPD platelets contain functional α-granule membranes.

Such studies on platelets from two α-SPD patients have recently been reported by Rosa et al. (21). Both the content and surface expression of GMP-140 were found to be similar to that in normal platelets, thus suggesting the presence of α-granule structures in these platelets despite the marked decrease in certain α-granule constituents. These studies also demonstrated that two constituents, IgG and albumin, which can be taken up into platelet α-granules from plasma via endocytosis (22), were present in substantial amounts and secreted from α-SPD plate-

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1. *Abbreviation used in this paper:* SPD, storage pool deficiency.

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lets. Based on these findings, it was therefore suggested that the α-granule abnormalities in α-SPD may result from a defect in the targeting of endogenously synthesized secretory proteins to developing α-granules in the megakaryocyte (21).

The present studies have examined the content and surface expression of GMP-140 in platelets from patients with αδ-SPD, including one patient with a severe deficiency of α-granule constituents (~ 20% of normal amounts), and three members of a family with lesser deficiencies (50–100% of normal) of α-granule substances. Surface expression of GMP-140 was assessed by flow cytometric measurement of the binding of GMP-140-specific monoclonal antibodies to activated platelets, and the content and localization of this antigen were determined using a specific ELISA assay and immunogold electron microscopy of frozen thin sections. Our results indicate that platelets from the patient with a severe α-granule deficiency, in marked contrast to both the αδ-SPD patients with lesser α-granule deficiencies and the α-SPD patients described previously, contain reduced amounts of GMP-140. Furthermore, they suggest that this reduction is associated with the presence of heterogeneous populations of platelets, containing either little or no GMP-140 or substantial-to-normal amounts of this protein.

Methods

Monoclonal antibodies. The monoclonal antibodies S12 and W40, directed against independent epitopes on GMP-140 (17, 23), were generously provided by Dr. Rodger P. McEver, Oklahoma Medical Research Foundation, Oklahoma City, OK. PAC1 and A_2A_9 are monoclonal antibodies directed against the platelet GP IIb–IIIa complex; PAC1 is specific for this complex on activated platelets (24), whereas A_2A_9 recognizes a complex-dependent epitope on both activated and unactivated platelets (25).

Patients. J.C. is a 45-y-old woman with a lifelong bleeding disorder in whom previous studies have disclosed combined deficiencies of dense granules, α-granules, and granule-bound substances (1). The ATP/ADP ratio in her platelets was 3.5 (normal 1.4–2.6) and the 5-hydroxytryptamine (5HT) content was 45 nmol/10^{11} platelets (normal 183–413), both indicative of a dense granule deficiency. Previously reported values (as percentage of the mean normal value) for α-granule substances were as follows: platelet factor 4 (PF4) = 11%, β-thromboglobulin (β-TG) = 19%, fibrinogen = 30%, platelet-derived growth factor (PDGF) was undetectable (1). In subsequent studies (unpublished), the von Willebrand antigen (vW:Ag) content of her platelets was 0.08 U per 10^9 platelets, 16% of that in normal platelets (26). The number of α-granules per square centimeter in electron micrographs of her platelets was ~ 22% of normal (1). In the majority of her platelets, α-granules were either entirely absent or markedly diminished. However, some platelets appeared to have a normal content of granules (1). In other studies, a markedly diminished initial aggregation response to epinephrine, with a normal number of α_2-adrenergic receptors, has also been found (8).

Family C. Three members (D.C., age 50 yr, and her children S.C. and T.C., ages 29 and 16 yr) of the originally reported family with a storage pool defect (27–29) were studied. Another of D.C.'s children (R.C., age 31 yr) was unavailable for inclusion in this study. Previously reported (1) studies on patients D.C., R.C., and S.C. have shown the following (means±SEM): ATP/ADP = 6.4±0.1, 5HT = 135±21 nmol/10^{11} platelets, platelet α-granule substances (percentage of normal): PF4 = 52±10%, β-TG = 74±17%, PDGF = 106±9%, fibrinogen = 36±7%; α-granules per square centimeter in electron micrographs was 78±2% of normal. Platelet vW:Ag values in patients S.C. and D.C. were 104% and 102% of normal, respectively. More limited studies on patient T.C., whose platelet aggregation defects are entirely similar to other family members, have disclosed a platelet ATP/ADP ratio of 7.3, and a 5HT value of 213 nmol/10^{11} platelets. Other platelet abnormalities in this family include a unique lipid defect (30), increased amounts of glycoprotein IV (31), and a decreased number of α_2-adrenergic receptors (8). The clinically affected father of patient D.C. (27) died of documented acute myelogenous leukemia at age 73 yr, and his father, who also had a lifelong bleeding disorder, was also said to have died of leukemia.

Flow cytometric analysis of S12, W40, and PAC1 binding to platelets. Monoclonal antibodies were conjugated with fluorescein isothiocyanate (FITC) for measurement of binding to stimulated and unstimulated platelets by flow cytometry using a FACStar® flow cytometer (Becton, Dickinson & Co., Salt Lake City, UT). The procedures for FITC labeling and the instrument conditions for antibody binding measurements were as described previously (32). Fluorescein/protein molar ratios of all antibodies ranged from 2 to 3. It has been shown previously that antibody-dependent platelet fluorescence is directly related to the number of antibody molecules bound per cell (32). Blood was collected into acid citrate dextrose anticoagulant and platelet-rich plasma (PRP) prepared. PGE_1 (1 μM, final concentration) was added to PRP and platelets were then gel-filtered into Ca-free Tyrode's buffer (33) containing 5 mM Hepes, 0.1% glucose, and 0.2% bovine serum albumin. For studies of FITC–S12 and FITC–W40 binding, 5 mM EDTA was added to the gel-filtered platelets. Gel-filtered platelets (5 μl) were incubated with 1 μM PGE_1, 5 U/ml bovine thrombin, or 0.2–0.5 μM phorbol myristate acetate (PMA), and the appropriate FITC-labeled MAb, in a total volume of 50 μl for 20 min at 22°C in the dark, and then diluted with 500 μl of the gel-filtration buffer. Samples from patient J.C. and two simultaneous controls were then transported from New York City to Philadelphia for assay of MAb binding. All but one study on the members of family C were carried out in Philadelphia; on one occasion patient S.C. was studied in New York and the samples transported to Philadelphia for assay. All transported samples were assayed within 4–6 h of sample preparation. On one occasion, MAb binding in platelets from patient J.C. and two simultaneous controls was measured in PRP instead of gel-filtered platelets. Since the results obtained were entirely comparable to those obtained in gel-filtered platelets, the combined results of all studies are presented. Similarly, stimulation by 0.2 and 0.5 μM PMA also produced comparable MAb binding, and these results are also combined.

Antigen-capture ELISA for total platelet GMP-140 content. Total platelet GMP-140 content was measured in Triton X-100 lysates of washed platelets. PRP containing 1 μM PGE_1 was centrifuged at 1,700 *g* for 15 min at room temperature, and the platelet pellet resuspended in 10 ml of 20 mM PIPES-0.15 M NaCl, pH 6.5, containing 1 μM PGE_1. The platelet suspension was centrifuged again as above, and the platelet pellet, at 2×10^9 platelets per milliliter, lysed in a buffer containing 50 mM Tris, 1% Triton X-100, 100 μM leupeptin, and 0.9 mM phenylmethylsulfonyl fluoride. The lysate was stirred for 30 min at room temperature, then centrifuged at 17,500 *g* for 10 min at 4°C, and the supernate decanted. Supernatants were assayed on the same day that blood was obtained.

The antigen-capture ELISA for GMP-140 developed by one of us (Dr. Shattil) was performed as described in detail previously (15). Briefly, microtiter wells were coated with antibody W40 overnight at 4°C, then blocked with 1% BSA plus 0.02% sodium azide in phosphate-buffered saline. Serial dilutions of the platelet lysates were added in triplicate for 2 h at room temperature. GMP-140 was detected by addition of biotin-conjugated S12 for 2 h at room temperature and quantitated by addition of streptavidin-conjugated horseradish peroxidase (30 min at room temperature) and 2,2′ azino-di-[3-ethylbenzthiazoline sulfonate] peroxidase substrate (15 min at room temperature). Absorbance was measured at 405 nm. GMP-140 was expressed as a percent of that obtained in one or two simultaneous controls.

Immunocytochemical electron microscopy for GMP-140. Aliquots of the platelet suspension in 20 mM PIPES-0.15 M NaCl containing 1 μM PGE_1, prepared as described above, containing 1×10^9 platelets were fixed in 8% paraformaldehyde in 0.1 M sodium phosphate buffer,

pH 7.4, at 4°C for 2–3 h and then washed in the same buffer containing 10% (wt/vol) sucrose (34). They were infiltrated for 30 min with 2.3 M sucrose, embedded in the sucrose solution, frozen, and stored in liquid nitrogen. The frozen thin-section techniques described by Tokuyasu (35) were used, with the modifications described by Griffiths et al. (36). When sections were incubated with polyclonal antibodies to GMP-140 (obtained from Dr. R. P. McEver), the probe was 5 nm colloidal gold conjugated with goat anti-rabbit IgG (Janssen Pharmaceutical, Beerse, Belgium). Control measures for all procedures included the substitution of buffer or pre-immune rabbit serum IgG for specific primary antibody.

Determination of platelet IgG content. Total platelet IgG was measured in Triton X-100–lysed concentrated platelet suspensions using a specific ELISA essentially as described by George et al. (22). Blood was collected into 1/10 vol of 1% EDTA in 0.7% saline and PRP was prepared. Platelets were pelleted from PRP by centrifugation at 2,500 *g* for 20 min at 20°C, resuspended in 1% ammonium oxalate to destroy contaminating red cells, centrifuged at 2,500 *g* for 15 min at 20°C, and washed three times in 0.05 M Tris-0.6% saline-0.1% EDTA. Before the last wash, platelets were counted, and then resuspended in a total volume of 350 μl. Platelet counts in the final resuspension ranged from 2 to 5 × 10^9 platelets per milliliter. An aliquot (100 μl) of this suspension was incubated with 10 μl of 10% Triton X-100 for 20 min at 37°C and then frozen at −80°C for assay of IgG. At least three dilutions of the lysed suspension were assayed, each in triplicate. The primary antibody, affinity-purified goat anti-human IgG(Fc) was from ICN Immunobiologicals, Lisle, IL, and the secondary antibody, affinity-purified biotinylated anti-human IgG (H + L) and the avidin-biotinylated horseradish peroxidase (Vectastain kit) were from Vector Laboratories, Burlingame, CA. IgG standards were obtained from Sigma Chemical Co., St. Louis, MO. The substrate was *o*-phenylene diamine (Sigma Chemical Co.); color development was stopped after 10 min by addition of 6N H_2SO_4 and absorbance read at 450 nm after 10 min.

Results

Surface expression of GMP-140. Expression of the α-granule membrane protein GMP-140 on the surface of maximally activated platelets from αδ-SPD patients and normal controls was assessed by flow cytometric measurement of the binding of FITC-S12 and FITC-W40, two monoclonal antibodies which recognize independent epitopes on GMP-140 (17, 23). Fig. 1 shows that seven- to eightfold increases in platelet-associated FITC–S12 and FITC–W40 fluorescence were induced by 5 U/ml thrombin or 0.2 or 0.5 μM PMA in platelets from nine controls used in these studies. The figure also shows that thrombin stimulation produced comparable increases in FITC–S12 binding in control platelets transported from New York to Philadelphia vs. nontransported control platelets. Transported and nontransported control platelets were also similar with respect to PMA-induced FITC–S12 binding and FITC–W40 binding induced by both agonists (data not shown).

Multiple studies on platelets from patient J.C., in which the number of α-granules and contents of α-granule constituents are ~ 20% of normal (1), showed a consistent and marked decrease in the surface expression of GMP-140 after stimulation. FITC–S12 binding induced by thrombin and PMA was 24.3±11.7 and 30.8±9.2% of the values obtained in simultaneous controls on four different occasions (Table I). FITC–W40 binding, measured on two occasions, was similarly decreased: 31.4 and 38.4% of simultaneous controls after thrombin and PMA stimulation, respectively.

Surface expression of GMP-140 was also measured in the stimulated platelets of three members of family C, in whom deficiencies of α-granules and α-granule constituents are less severe than those in J.C., generally ranging from 50 to 100% of normal values (1). FITC–S12 and FITC–W40 binding measured on multiple occasions were consistently greater than that in patient J.C., varying from 68.0±10.8 to 94.6±28.9% of simultaneous control values. W40 binding, measured on one occasion only, was somewhat lower than the mean values of S12 binding (Table I), but entirely similar to the S12 binding values obtained in the same study in all three patients (data not shown).

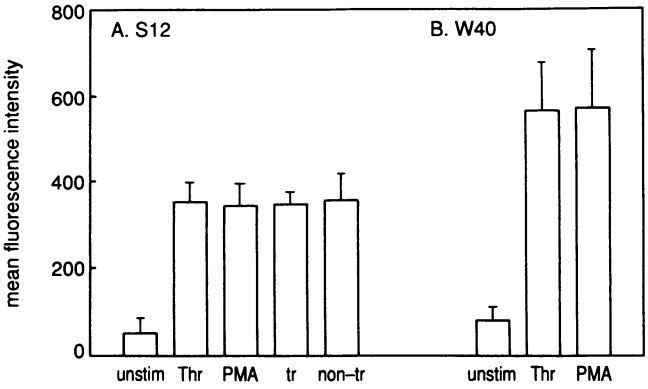


Figure 1. Mean fluorescence intensity values (arbitrary units) of platelet-associated FITC-S12 (*A*) and FITC-W40 (*B*) in unstimulated (*unstim*), thrombin (*Thr*), and PMA-stimulated control platelets measured by flow cytometry. Thrombin concentration was 5 U/ml and PMA concentration was 0.2 or 0.5 mM. Similar values were obtained at both PMA concentrations and the results were therefore combined. FITC-S12 fluorescence intensities are also shown for thrombin-treated control platelets transported from New York to Philadelphia (*tr*, $n = 4$) vs. nontransported control platelets (*non-tr*, $n = 5$). Bars represent means±S.D. for $n = 9$ (S12) and $n = 4$ (W40) controls.

Table I. FITC–S12 and FITC–W40 Binding Induced by Thrombin (5 U/ml) and PMA (0.2 or 0.5 μM) in Platelets from αδ SPD Patients

	FITC–S12 binding		FITC–W40 binding	
	Thrombin	PMA	Thrombin	PMA
	percentage of simultaneous controls			
J.C.	24.3±11.7 (4)	30.8±9.2 (4)	31.4 (2)	38.4 (2)
Family C				
D.C.	94.6±28.9 (3)	80.6±15.8 (3)	67.3 (1)	63.8 (2)
S.C.	85.9±23.0 (5)	79.1±9.4 (5)	69.3 (1)	71.5 (1)
T.C.	76.3±18.0 (3)	68.0±10.8 (3)	61.8 (1)	58.1 (1)

Binding is calculated as the mean fluorescence intensity of FITC-labeled antibody associated with platelets in stimulated samples minus that in unstimulated samples, the latter containing 1 μM PGE_1. For each patient, binding is expressed as the mean±SD percentage of that in simultaneous controls, for the number of observations indicated in parentheses.

Platelet activation assessed by PAC1 binding. Since S12 and W40 binding depends upon the activation-induced translocation of GMP-140 to the platelet plasma membrane, we also examined whether defects in activation might be responsible for decreased expression of GMP-140 on the platelet surface. Activation was assessed by measuring the binding of FITC-PAC1, a monoclonal antibody which recognizes an epitope on the fibrinogen receptor associated with glycoprotein IIb-IIIa in activated, but not unactivated, platelets (24). PMA-induced FITC-PAC1 binding to platelets of patient J.C. was 77±14% of that in simultaneous controls (Table II), a value which is not significantly reduced compared to the binding in normal platelets. Furthermore, the ratio of PMA-induced PAC1 binding to A_2A_9 binding, the latter representing total GPIIb-IIIa complexes on the unstimulated platelet plasma membrane (25), was similar in J.C. (1.37) and in two simultaneous controls (1.43, 1.45), indicating a similar degree of fibrinogen receptor exposure with this agonist. Thus, with PMA, the decreased surface expression of GMP-140 in patient J.C. cannot be attributed to an impairment of platelet activation.

FITC-PAC1 binding induced by thrombin was measured in J.C.'s platelets on one occasion, and was substantially decreased compared to the values obtained in two simultaneous controls (Table II). In addition, PAC1 binding induced by 100 μM ADP was also decreased on this same occasion (37 and 38% of simultaneous control values), and that induced by thrombin (0.5 U/ml) plus collagen (10 μg/ml) measured in a separate study was 19% of the control value. Thus, with thrombin, an impairment of platelet activation may also contribute to the decreased surface expression of GMP-140 in this patient. The observation that PAC1 binding was reduced in response to physiological agonists which are believed to interact at specific membrane receptors, but not with PMA, which is generally held to circumvent agonist-membrane receptor mediated signal transduction pathways, suggests that the activation defect in J.C.'s platelets may be related specifically to membrane receptor-mediated events.

FITC-PAC1 binding induced by thrombin was similar to normals in patients D.C. and T.C., but was decreased in patient S.C., on one occasion, whereas PMA-induced PAC1 binding was the same as that in controls in all three members of family C (Table II).

Table II. FITC-PAC1 Binding Induced by Thrombin and PMA in Platelets from αδ SPD Patients

	FITC-PAC1 binding	
	Thrombin	PMA
	percentage of simultaneous controls	
J.C.	32.4, 36.8	77.4±13.9 (6)
Family C		
D.C.	87.2, 81.2	90.8, 93.2
S.C.	48.3, 57.6	83.5±18.4 (3)
T.C.	92.2, 85.9	90.1, 92.5

Values shown are percent of that in each of two simultaneous controls measured on one occasion or means±SD for the number of observations indicated in parentheses. Thrombin and PMA concentrations are the same as in Table I.

Heterogeneity of S12 and W40 binding in platelets of patient J.C. Examination of the distributions of FITC-S12 and FITC-W40 fluorescence intensities in unstimulated and stimulated platelets from patient J.C. revealed the presence of heterogeneous populations of platelets with respect to antibody binding. Both unstimulated and thrombin-stimulated control platelets appeared as single populations in the fluorescence intensity histograms, as did unstimulated platelets of patient J.C. (Fig. 2). However, with both antibodies, at least two populations of this patient's thrombin-stimulated platelets were evident (Fig. 2). A similar pattern was also seen in the histogram of this patient's PMA-stimulated platelets (data not shown). The percentage and mean fluorescence intensities of stimulated cells in each population were estimated from the histograms by setting a marker at the upper edge of the unstimulated platelet peak and determining these parameters in stimulated cells to the left and right of this marker, and are shown in Table III. The major population comprised ~ 80–85% of total platelets, and had mean fluorescence intensity values only slightly greater than those of unstimulated platelets, and similar to the values obtained in normal unstimulated cells, indicating that this population bound little or no S12 or W40. The minor population comprised 15–20% of total platelets, with mean fluorescence intensity values comparable to those in normal stimulated platelets, suggesting that this population expressed normal amounts of GMP-140 on the platelet surface.

In contrast, stimulated platelets from all three members of family C showed no evidence of distinct populations differing

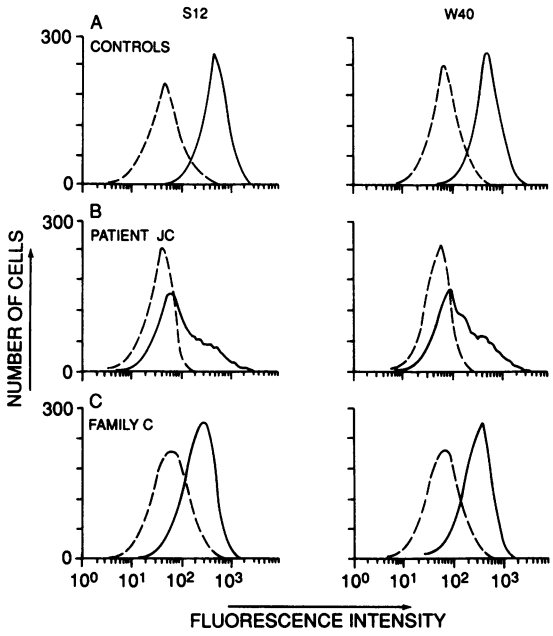


Figure 2. Histograms of cell number vs. fluorescence intensity for unstimulated (*dashed line*) and thrombin (5 U/ml)-stimulated (*solid line*) gel-filtered platelets incubated with FITC-S12 (*left panels*) or FITC-W40 (*right panels*). Results shown were obtained from a normal control (*A*), patient J.C. (*B*), and patient T.C. from family C (*C*). Patterns obtained in the other two members of family C were similar to that shown for T.C. The histograms illustrate the distribution of fluorescence intensities measured in 10,000 cells.

Table III. Estimated FITC-S12 and FITC-W40 Binding, Expressed as Mean Fluorescence Intensities, in Subpopulations of Stimulated Platelets from Patient J.C.

	Mean fluorescence intensities	
	FITC-S12	FITC-W40
Unstimulated platelets		
J.C.	27±8 (4)	61 (2)
Controls	36±9 (4)	94 (2)
Thrombin-stimulated platelets		
J.C. major population (85±2%)	60±20 (3)	121 (2)
minor population (15±2%)	579±188	1031
Controls	489±95 (4)	883 (2)
PMA-stimulated platelets		
J.C. major population (79±6%)	56±27 (3)	87 (2)
minor population (21±6%)	595±203	1075
Controls	511±92 (4)	913 (2)

FITC-S12 and FITC-W40 values shown are the means±SD of the platelet-associated mean fluorescence intensities for the number of observations indicated in parentheses. Mean fluorescence intensities of stimulated samples are calculated as the difference between stimulated and unstimulated values. The mean±SD percentage of the total platelet population is also given for the major and minor populations of patients J.C.'s platelets. See text for the method of distinguishing the major and minor populations. Thrombin and PMA concentrations are the same as in Table I.

Table IV. Total Platelet Content of GMP-140 in Triton X-100 Lysates of Unstimulated Platelets Measured by Antigen-capture ELISA

	Total platelet GMP-140
	percentage of control values
J.C.	49±8 (6)
Family C	
D.C.	107±7 (4)
S.C.	
1	98±37 (5)
2	115±25 (4)
T.C.	102±13 (4)

Values shown are the mean±SD percentages of the optical densities obtained in simultaneously-measured normal subjects, determined from the number of points on the linear portion of the optical density vs. platelet concentration assay curve shown in parentheses. All values except SC 2 were determined vs. two simultaneous controls; SC 2 was determined vs. one control.

in S12 and/or W40 binding (histograms of T.C. only shown in Fig. 2).

Total platelet content of GMP-140. To assess the total platelet content of GMP-140 independently of platelet activation, an antigen-capture ELISA technique was utilized to determine GMP-140 content in Triton X-100 lysates of unstimulated platelets. Assay curves were generated using serial dilutions of lysates from patient platelets and simultaneously prepared lysates from control platelets, and GMP-140 content was expressed as mean (±SD) percentage of controls, obtained from values on the linear portions of the assay curves. Total GMP-140 content of patient J.C.'s platelets was 49±8% of that in control platelets (Table IV), thus indicating that the decreased surface expression of GMP-140, as measured by flow cytometry, is associated with a decreased platelet content of this antigen. In contrast, normal amounts of total platelet GMP-140 were measured in platelets from each of the three members of family C.

The same samples that were used for the ELISA measurements of GMP-140 in patient J.C. and two simultaneous controls were also analyzed for GMP-140 protein by quantitative radioimmunoassay, kindly performed by Dr. Kevin Moore of the University of Oklahoma. The platelet lysate from patient J.C. contained 0.43 ng of GMP-140/μg of platelet protein, compared to values of 0.92 and 1.14 ng GMP-140/μg of platelet protein in the control platelet lysates. Thus, the decreased content of GMP-140 protein in this patient's platelets found by quantitative radioimmunoassay is entirely comparable to that obtained using the ELISA assay (Table IV).

Immunogold staining of GMP-140 in platelet frozen thin sections. The content and subcellular localization of GMP-140 in unstimulated platelets was examined further immunocytochemically using an IgG-immunogold probe. Frozen thin sections of unstimulated platelets were incubated with polyclonal antibodies to GMP-140, and antibody localization detected by electron microscopy using an immunogold probe as described previously (12, 21). Platelets from patient J.C. were also found to be heterogeneous with respect to GMP-140 content by this technique.

One population, comprising ~ 20% of total platelets, was entirely devoid of GMP-140 immunogold label and of typical α-granules (Fig. 3 *A*) or contained only very small amounts of the immunogold label (Fig. 3 *B*). In the majority of this patient's platelets (~ 60%), the GMP-140 label was present in reduced amounts, associated predominantly with the membranes of clear vacuoles (Fig. 4 *A*) and occasionally with granules. However, there were also many vacuoles which did not contain the immunogold label (Fig. 4 *A*). A similar distribution of immunogold label among clear vacuoles and occasional granules, but with normal amounts of GMP-140, was observed previously in the gray platelet syndrome (21). In the remaining 20% of patient J.C.'s platelets, GMP-140 was present in amounts similar to or only slightly less than normal, and was localized to both typical α-granules and the membranes of clear vacuoles (Fig. 4 *B*).

Such heterogeneity in immunogold labeling was not evident in platelets from the three members of family C. Thin sections of these platelets did show some variation in the numbers of typical α-granule structures, which appeared to be relatively normal in some cells (Fig. 5 *A*), but decreased in others (Fig. 5 *B*). However, in both cases GMP-140 was detected in these typical α-granule structures, as well as in clear vacuoles. Thus, in these patients, platelets containing little or no GMP-140 were not observed.

Platelet IgG content. IgG has been shown to be an α-granule constituent in human platelets (22). In contrast to platelet factor 4, which is synthesized only in megakaryocytes (37) and thus incorporated into α-granules endogenously, IgG, like the plasma proteins albumin (21, 38) and fibrinogen (38, 39), appears to be incorporated into the granules via endocytosis from plasma (21, 22, 38). IgG was found to be present in platelets of

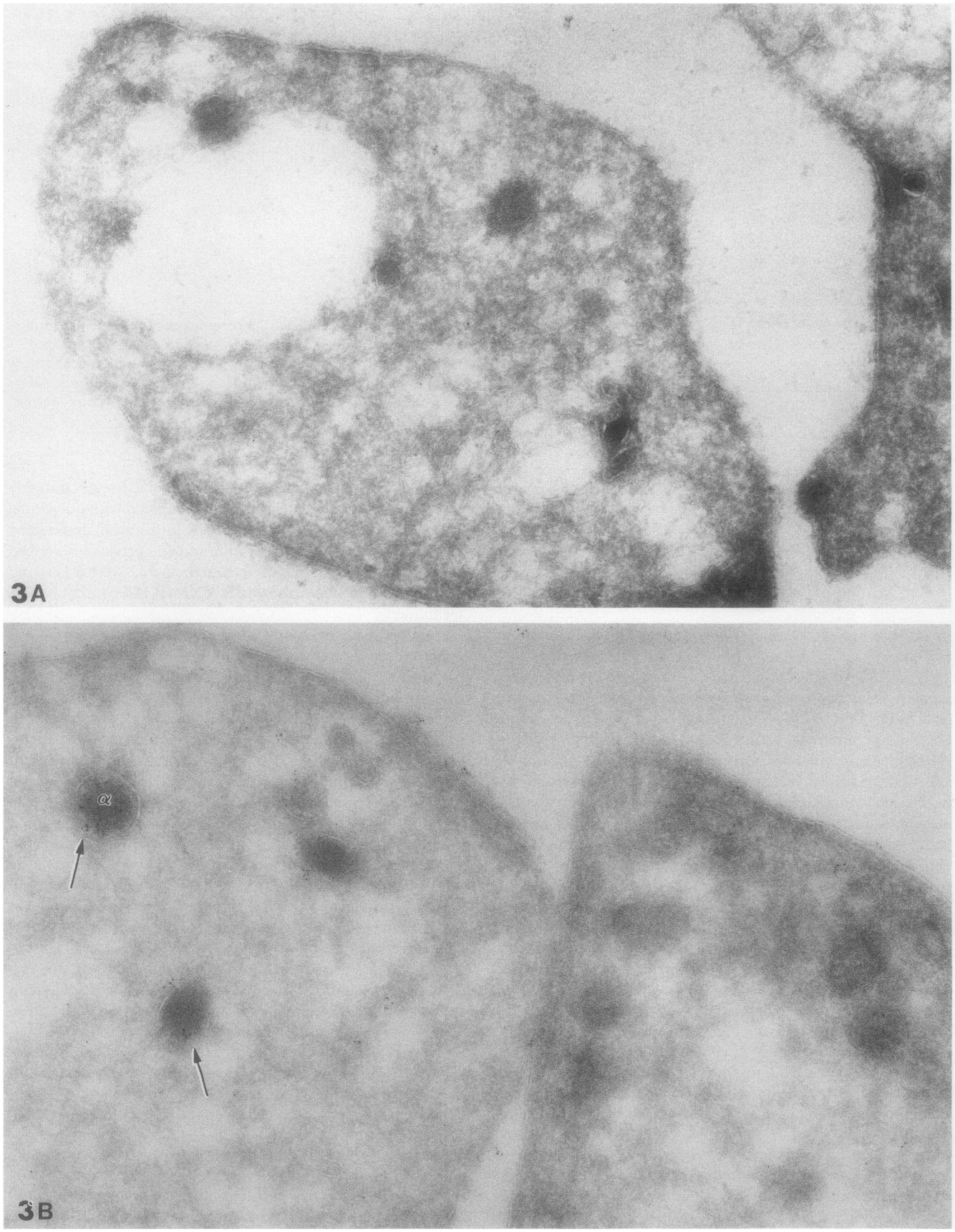
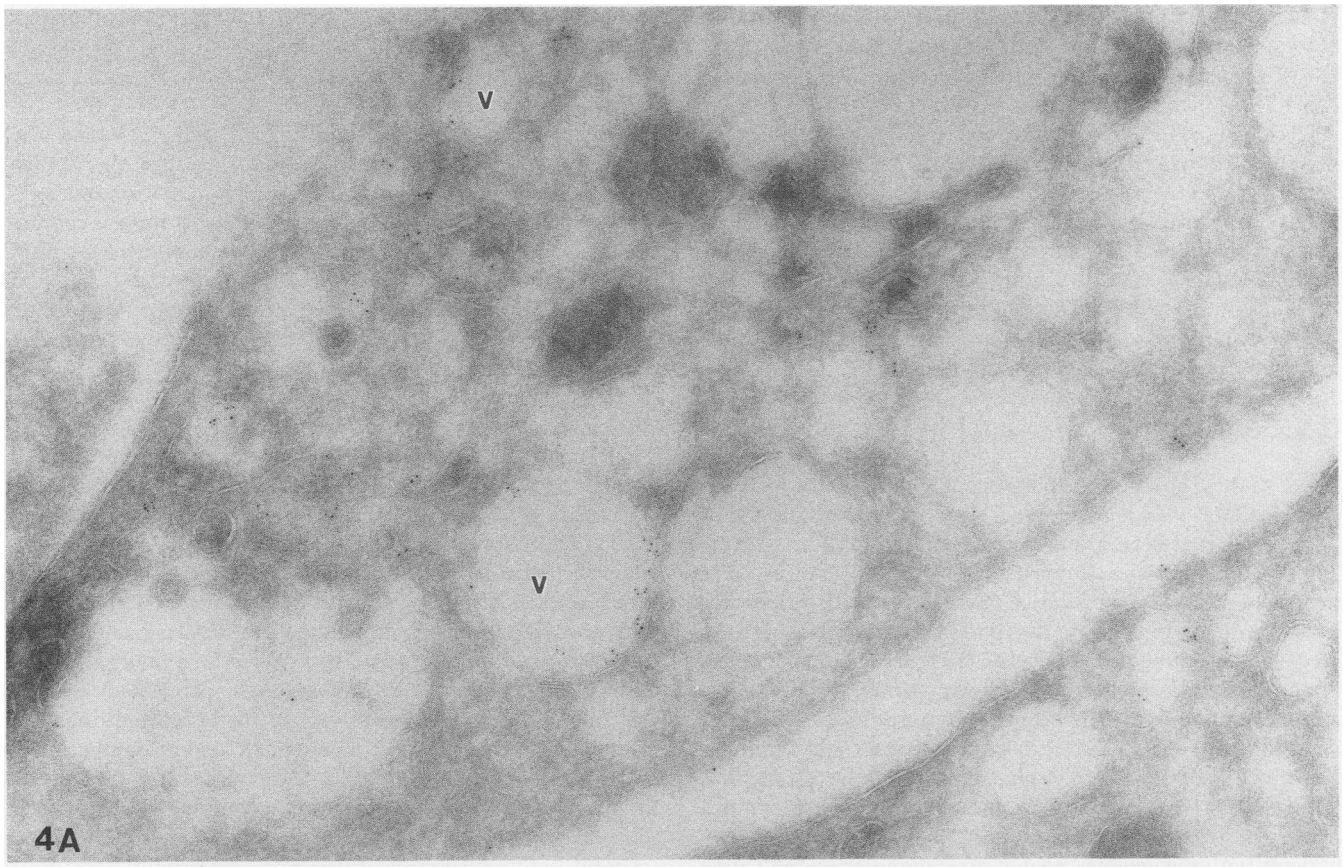
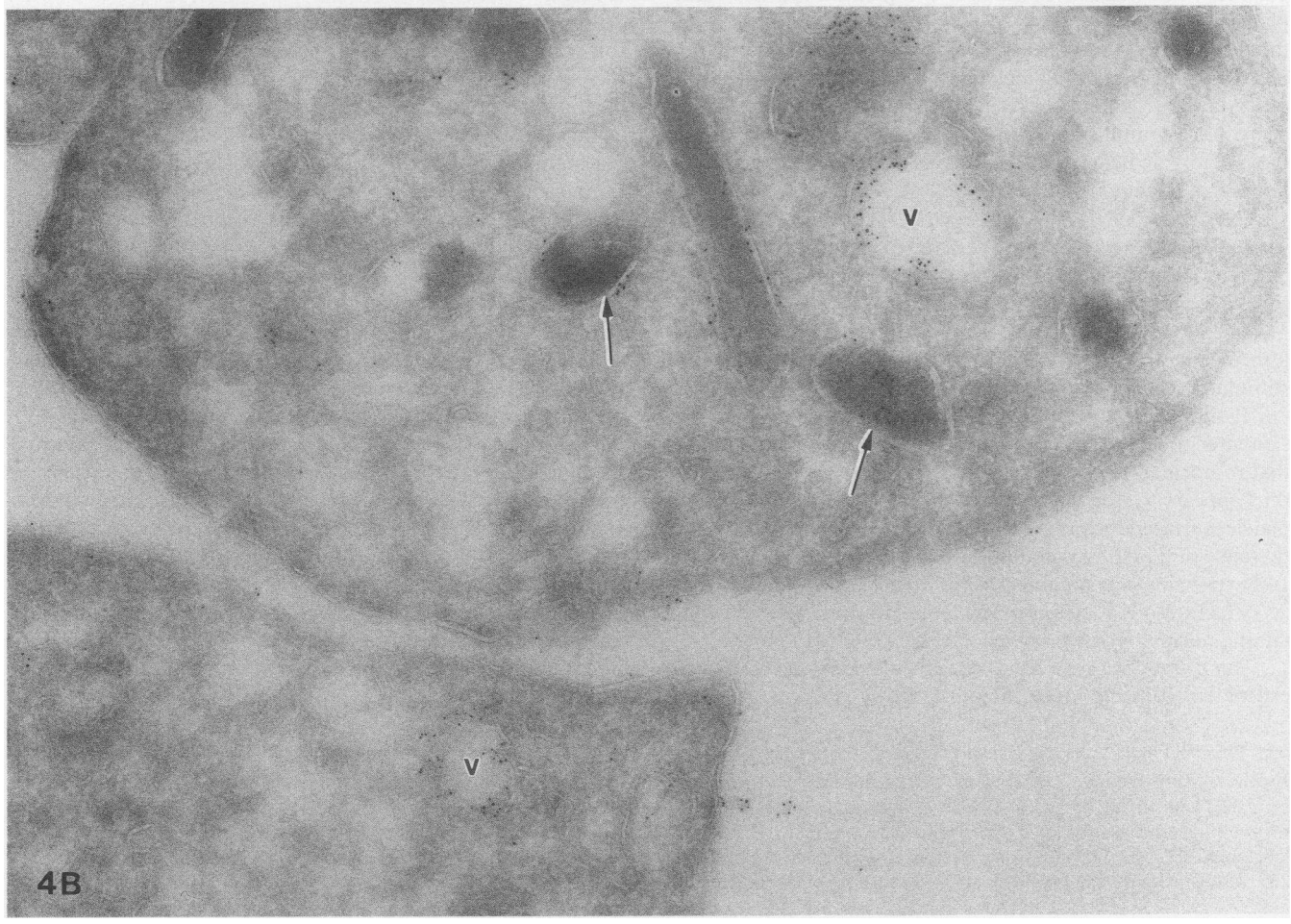


Figure 3. Immunogold labeling of GMP-140 in frozen thin sections of platelets from patient J.C. A platelet virtually devoid of typical α-granules and of the GMP-140 immunogold label is shown in *A* and platelets containing only occasional α-granules (α) or GMP-140 immunogold label (*arrows*) are shown in *B*. Platelets with this type of labeling were estimated to comprise ~ 20% of this patient's total platelets. (*A*) ×48,000; (*B*) ×58,000.



4A



4B

patients with the gray platelet syndrome in substantially greater amounts than were the endogenously synthesized constituents (21). Total IgG in platelets of patient J.C. was 1.9 fg per platelet (Table V). This value was below the lower limit of the range of values obtained in 11 normal subjects, but was not significantly decreased compared to the normal mean. This amount of IgG represented 43% of the normal mean value, and was thus about twofold greater than the amounts of the endogenous constituents (approximately 20% of normal) measured previously (1).

Platelet IgG content among the members of family C ranged from 2.2 to 2.9 fg per platelet; these values were also not significantly decreased, and were within the range of values obtained in the normal subjects (Table V).

Discussion

These studies show that the platelet content and surface expression of GMP-140, a membrane glycoprotein specific for α-granules in platelets, are decreased in a patient with severe combined deficiencies of dense granules and α-granules. The decreased surface expression of GMP-140 was demonstrated by the reduced binding of two independent anti–GMP-140 monoclonal antibodies (S12 and W40) to maximally stimulated platelets as measured by flow cytometry, and the decreased content of GMP-140 by both antigen-capture ELISA measurements of GMP-140 in platelet lysates and immunogold labeling of GMP-140 in platelet frozen thin sections. The decreased GMP-140 content in this patient is in clear contrast to the findings of normal amounts of platelet GMP-140 in patients with deficiencies specific to α-granules (α-SPD, gray platelet syndrome) reported by Rosa et al. (21), as well as in the three patients with less severe deficiencies of α-granules combined with dense granule deficiencies studied here. Thus, the observation of decreased GMP-140 and hence, by inference, of decreased α-granule membranes in this patient (J.C.) illustrates the phenotypic heterogeneity of platelet α-granule deficiencies in man.

Histograms of FITC-S12 and FITC-W40 fluorescence intensity (Fig. 2) revealed the presence of at least two distinct populations of this patient's stimulated platelets with respect to surface expression of GMP-140. The majority of her platelets, ~ 80–85% of the total cells, were estimated to have a mean fluorescence intensity only slightly greater than that of unstimulated cells, indicating little or no surface expression of GMP-140, while the remaining 15–20% of her platelets were estimated to have a mean fluorescence intensity comparable to that in normal stimulated platelets. This latter subpopulation thus appears to express normal amounts of GMP-140 on the platelet surface following stimulation. Further evidence for the presence of this subpopulation of platelets with normal GMP-140 expression was obtained from the immunogold EM studies, which also revealed a minor population of platelets containing normal or near-normal amounts of GMP-140.

The number of platelets containing little or no GMP-140 estimated from the immunogold labeling studies, however, comprised only 20% of this patient's total platelets, compared to the 80–85% with little or no surface expression of GMP-140 indicated by the flow cytometry studies. Thus, it seems likely that this latter population includes platelets which contain decreased, but detectable, amounts of GMP-140, but which may not fully express this protein on the platelet surface after activation. Such a hypothesis would also be consistent with the observation that total GMP-140, as measured by antigen-capture ELISA (Table IV), was somewhat greater than the maximum amount expressed on the platelet surface following stimulation (Table I). Measurements of PAC1 binding (Table II) indicate that impaired platelet activation may contribute to this difference in surface expression vs. content of GMP-140 in thrombin-stimulated platelets, but probably does not do so in PMA-stimulated platelets. This discrepancy between surface expression and total content of GMP-140 may arise, therefore, at least in part, from the granule abnormality itself. For example, localization of GMP-140 by immunogold probe in patient J.C.'s platelets, as in the studies of gray platelet syndrome (21), showed that this protein was associated predominantly with clear vacuoles of varying sizes. In the gray platelets, these vacuoles were presumed to represent the abnormal α-granule structures in which the endogenously synthesized protein constituents (platelet factor 4, β-thromboglobulin, von Willebrand factor, platelet-derived growth factor, thrombospondin) were absent (21). It may be that in patient J.C., some or all of these vacuoles containing GMP-140 do not fuse with the plasma membrane upon stimulation, thus accounting for the greater decrease in surface expression of GMP-140 than in GMP-140 content.

Our findings that GMP-140 is decreased in the majority of this patient's platelets suggest an abnormality in, or absence of, the α-granule structures themselves; thus the defect responsible for this α-granule storage deficiency is not likely to be the same as that postulated for the gray platelet syndrome, i.e., an impairment in the packaging of endogenously synthesized platelet proteins into developing α-granules in the megakaryocyte (21). This conclusion is further supported by the difference found in the ability of J.C.'s platelets vs. platelets from patients with the gray platelet syndrome, to accumulate IgG, an α-granule constituent which is taken up into the granules via endocytosis rather than via endogenous synthesis in the megakaryocyte (21, 22, 38). This difference is most striking when the platelet contents of IgG are expressed relative to the contents of endogenous α-granule substances: in patient J.C. IgG content was 43% of the normal mean (Table V) vs. contents of ~ 20% of normal for endogenous substances (1), whereas in the gray platelet syndrome, platelet IgG was found to be 68% of the normal mean vs. undetectable levels of the endogenous constituent platelet factor 4 (21). Thus, the accumulation of IgG relative to the contents of endogenous α-granule constituents is markedly less in J.C.'s platelets than in gray platelets, and this result would seem to be more consistent with a defect in the granule structure itself than with a defect in mechanisms of packaging constituents into the granule.

Figure 4. (*A*) Immunogold labeling of frozen thin sections representing the majority, ~ 60%, of platelets from patient J.C. The label is associated primarily with the membranes of clear vacuoles (*v*), although there are also many vacuoles which are not labeled for GMP-140; ×58,000. (*B*) Frozen thin sections of platelets from patient J.C., which are most similar to normal platelets with respect to α-granule content and immunogold label for GMP-140. The immunogold label is associated with both typical α-granule structures (*arrows*) and clear vacuoles (*v*). Approximately 20% of this patient's total platelets showed this type of labeling; ×58,000.

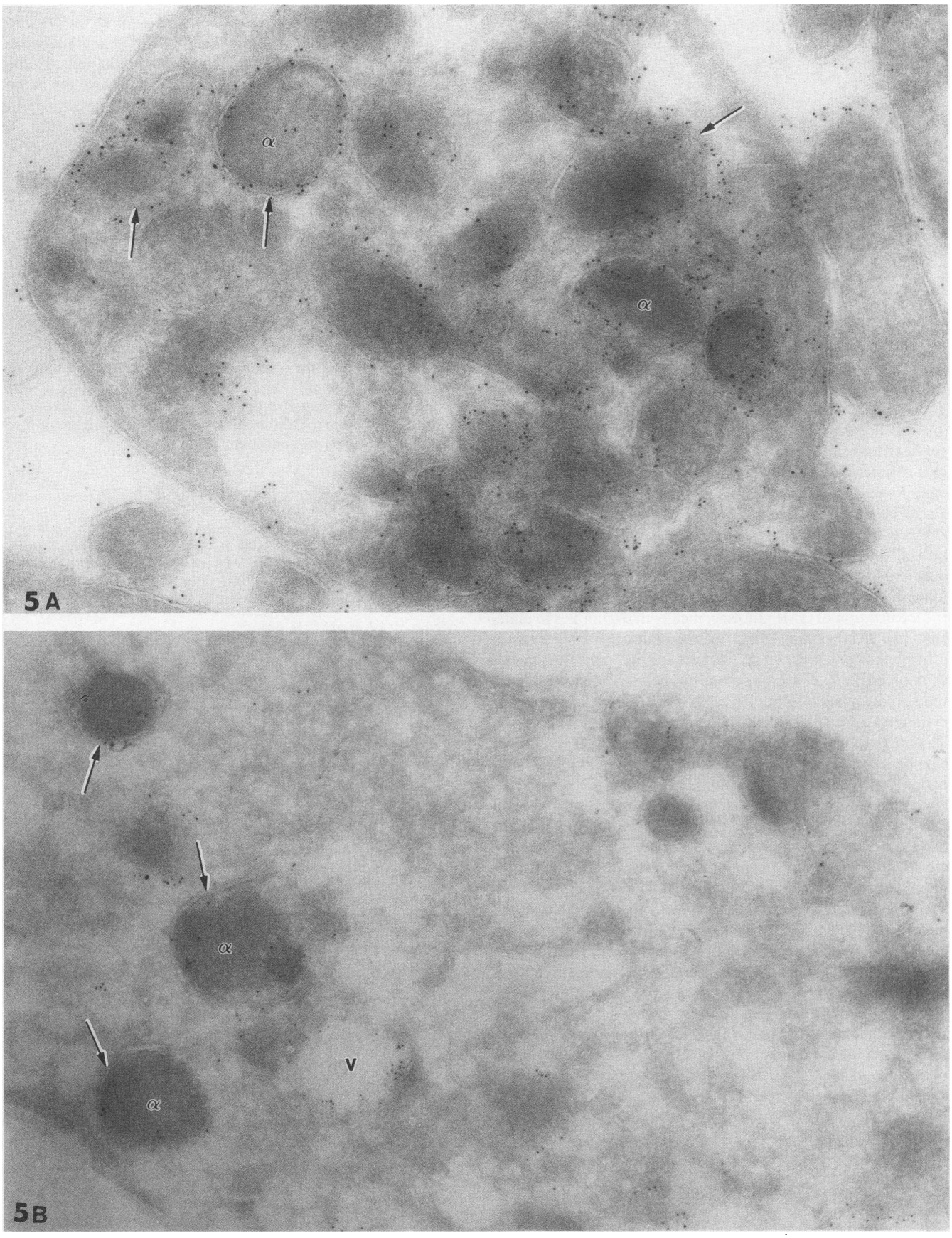


Figure 5. Immunogold labeling of GMP-140 in thin sections of platelets from family C. (*A*) A platelet from patient D.C. shows numbers of typical α-granules (α) and amounts of immunogold label (*arrows*) that are indistinguishable from that seen in normal platelets (cf. references 12 and 21). (*B*) A platelet from patient T.C. showing somewhat reduced numbers of typical α-granules (α), but immunogold labeling of both the α-granules (*arrows*) and some clear vacuoles (*v*). Similar variations in the number of typical α-granules, but not in the GMP-140 immunogold label associated with the granules and vacuoles, were seen among the platelets from each of the members of family C. (*A*) ×76,000; (*B*) ×64,000.

Table V. Total Platelet Content of IgG Measured by ELISA

	IgG content
	femtograms/platelet
J.C.	1.9
Family C	
D.C.	2.2
S.C.	2.4
T.C.	2.8
Controls (n = 11)	
Mean±SD	4.4±1.9
Range	2.2–7.7

Values shown are the contents of IgG in Triton X-100 lysates of concentrated platelet suspensions, with platelet counts ranging from 1.5 to 5 × 10^9 platelets per ml. IgG was assayed by a modification of an ELISA assay (reference 22), as described in Methods.

The decrease in GMP-140 in this patient's platelets also suggests that the defect in granule formation or structure occurs in her megakaryocytes. It is unlikely that this is due specifically to an absolute impairment in the capacity of her megakaryocytes to synthesize this protein, since normal amounts of GMP-140 and typical α-granules were found in a sub-population of her platelets. In addition, her platelets are also relatively deficient in dense granules, and evidence obtained from subcellular fractionation and differential secretion studies supports the localization of GMP-140 specifically in α-granules in platelets (13). It is generally held that granules in platelets, as in other secretory cells, are formed in the megakaryocytes through budding of vesicles from the Golgi complex, and that the several types of granules observed in neutrophilic leukocytes may arise at different stages of cell maturation (40, 41). The normal contents (1) and maximal secretable levels (42) of lysosomal acid hydrolases in patient J.C., as well as in the other patients with αδ-SPD (1, 42, 43), suggest that the formation of lysosomes during megakaryocyte development is normal, and that some defect in the Golgi complex or other aspects of the regulated secretory pathway (44), perhaps at a later stage of megakaryocyte development, may account for the defective α-granule and dense granule formation. It remains to be established whether the heterogeneity of the α-granule defect in the platelets of patient J.C. reflects a similar heterogeneity among megakaryocytes, or results from non-homogeneity of the territories from which platelets arise in individual megakaryocytes.

In contrast to that in patient J.C., the α-granule storage deficiency in the members of family C with αδ-SPD was not associated with a decreased platelet content of GMP-140. There was, however, a considerable degree of variability in the expression of GMP-140 on the platelet surface among these patients, as shown by the data in Table I. It is not at all clear at present how or whether this variability may be related to the considerable variations also evident in the contents of the different α-granule constituents in these patients' platelets, or to the type of underlying defect. The presence of normal total contents of GMP-140 may indicate that the α-granule deficiencies in this family, as in the gray platelet syndrome (α-SPD), do not arise from defective α-granule membrane formation, but rather from impairments in the processes by which constituents are sequestered within the granule structures.

In both family C and patient J.C. with αδ-SPD, the presence of a common defect responsible for both the α-granule and dense granule abnormalities is an attractive hypothesis. Studies similar to those described here to determine whether or not dense granule membrane elements are present in these patients would, therefore, be extremely useful, but are currently not possible. However, the recent identification of an immunoreactive protein which appears to be localized to platelet dense granules (45) raises the possibility that such studies may become feasible, in the near future.

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